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13. ABSTRACT *(Maximum 20*

Metastasis to bone is a major cause of morbidity from prostate cancer. Parathyroid hormone-related protein (PTHrP) is expressed by prostate cancer cells and stimulates osteoclasts. My project was **to test whether an antibody against PTHrP will block prostate cancer metastasis to bone.** I cloned the light and heavy chain cDNAs from a hybridoma, which secretes a PTHrP-neutralizing antibody which blocks breast cancer metastasis to bone but is untested against prostate cancer. The cDNAs in a bicistronic expression vector produced mouse IgG when transfected into human 293 cells. Secreted mouse antibody inhibited osteoclast formation and bone resorption in two tissue culture assays stimulated with PTHrP. The DNA was transfected into the human prostate cancer cell line PC3, which secretes PTHrP and causes osteolytic metastases. No stable clones could be obtained. Instead, PC3 clones with decreased PTHrP consequent to a dominant negative TGFbeta receptor were tested for their ability to metastasize to bone in animals. Paradoxically, bone metastases were made worse by the dominant negative receptor. This may be due to effects on IGFBP3 expression by PC3 cells. In my fellowship I learned many molecular and immunological techniques in the first year. In the second year I learned animal techniques related to bone metastases, including quantitative x-ray image analysis and skeletal histomorphometry.

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Targeted Antibody Therapy to Inhibit Bone Metastases by Prostate Cancer

Army Prostate Cancer Program 2 year Postdoctoral Fellowship

Abstract:

Metastasis to bone is a major cause of morbidity and mortality from prostate cancer. Most men dying from the disease have skeletal metastases, which cause abnormal bone growth, resulting in bone pain, fracture, and nerve compression. Prostate cancer prefers to grow in bone, since bone matrix stores large quantities of immobilized growth factors, which can stimulate tumor cells. When cancer cells produce factors which stimulate bone resorption, metastasis to bone is enhanced: tumor cell products stimulate cells called osteoclasts to release growth factors from the bone matrix. These products in turn stimulate the growth of tumor cells. Tumor-secreted factors capable of stimulating bone resorption are thus prime candidates to contribute to the establishment of bone metastasis. Parathyroid hormone-related protein (PTHrP) is known to be expressed by prostate cancer cells and is a major stimulator of osteoclasts. My project is **to test whether an antibody against PTHrP will block prostate cancer metastasis to bone**. I will use a gene expression vector to introduce the inhibitory antibody expression directly into the prostate cancer cells. This will be a **test to target inhibitors directly to the site of metastasis, which could be a useful approach for future gene therapy**.

In the first year of the work proposed I cloned and sequenced the light and heavy chain cDNAs from the 3F5 hybridoma, which secretes a monoclonal antibody directed against the N-terminal region of PTHrP. This antibody blocks breast cancer metastasis to bone but has never been tested against prostate cancer. The two antibody chain cDNAs were transferred into a bicistronic expression vector, producing mouse IgG antibody when the DNA was transiently transfected into human 293 cells in vitro. The secreted antibody potently and completely inhibited osteoclast formation and bone resorption in two tissue culture assays stimulated with 20ng/mL PTHrP (1-34) peptide. The DNA is presently in a vector designed for gene therapy.

In the second year I transferred the antibody-coding portion of this DNA into a vector, pcDNA3neo, known to be able stably to transfect cancer lines. This DNA will be transfected into the human prostate cancer cell line PC3, which our laboratory has shown to secrete abundant PTHrP and reliably to cause osteolytic metastasis in an animal model. PC3 cell subclones were to be selected which stably neutralize, via the transfected antibody, the PTHrP which they also secrete. These cells were to be tested for their ability to metastasize to bone in animals, which was expected to be greatly reduced compared to control PC3 cells. After much work, I found that the antibody ciould not be stably expressed in PC3 cells. An alternate project to reduce PTHrP expression by stable transfection with a dominant negative TGFbeta type II receptor subunit was developed. This experiment has now been successfully completed and reveals a novel role for insulin-like growth factor [IGF] derived from bone and tumor-produced IGF binding protein 3 [IGFBP3] in the regulation of prostate cancer growth in sites of bone metastases.

In the two years of my fellowship training I have learned many molecular and immunological techniques. I have and will present my results at the annual meetings of American Society of Bone & Mineral Research in Toronto, Phoenix and San Antonio. In the second year of my project I learned many animal techniques related to bone metastases, including quantitative x-ray image analysis and skeletal histomorphometry.

Targeted Antibody Therapy to Inhibit Bone Metastases by Prostate Cancer

Introduction:

Cancer of the prostate is the second leading cause of male deaths from cancer in the United States (Karp et al., 1996). The disease resulted in about 40,000 deaths in 1996. Up to 84% of patients dying of prostate cancer have been found to have bone metastases at autopsy (Jacobs, 1983). The metastases are predominantly osteoblastic, resulting in net gain of bone. The new bone formation is often disorganized and, particularly in the spine, can result in nerve compression syndromes, as well as bone pain. Mean survival from the time of diagnosis of bone involvement is 40 months. The frequency of prostate cancer metastasis to bone and the long duration of the disease result in a major contribution to patient morbidity from osteoblastic metastases (Coleman, 1997). However, the mechanisms responsible for prostatic cancer cell growth at metastatic sites in bone are incompletely understood.

Role of bone resorption in prostate cancer metastasis. Serum markers of bone resorption are elevated in prostate cancer metastatic to bone, suggesting that the bone lesions contain a lytic component associated with the blastic lesions. Bone resorption may release a variety of growth-regulatory factors, previously immobilized in the bone matrix, which can in turn stimulate the local growth of tumor cells. Thus, prostatic metastasis to bone may involve ongoing bone turnover, rather than requiring bone resorption only for the initial establishment of the metastatic foci (Goltzmann, 1997). Such a mechanism, linking metastasis by cancer cells with osteolytic bone resorption, has been experimentally established for human breast and lung cancers (Guise, 1997). The laboratory I am working in has established that PC3 prostate cancer cells cause very similar osteolytic metastases. All of these osteolytic tumor types secrete parathyroid hormone-related protein, PTHrP.

PTHrP and prostate cancer. Prostate cancer was first reported to express PTHrP by Iwamura et al. (1993). The human PC3 cell line secretes substantial amounts of PTHrP under serum-free conditions. However, PTHrP appears not to be an autocrine growth factor for normal prostatic cells (Peehl et al., 1997). Signaling through the G protein-coupled PTH/PTHrP receptor is not generally mitogenic (Guise & Mundy, 1996), but Dougherty et al. (1999) recently demonstrated growth dependence of prostate cancer on intracellular PTHrP. It has also been reported that the expression of PTHrP, assayed immunohistochemically, increased with increasing tumorigenic progression of prostate cells (Asadi et al., 1996). Thus the roles of PTHrP in prostate cancer growth may be complex. The group in which I am training has demonstrated that neutralizing antibodies against PTHrP can effectively decrease bone metastases (Guise et al., 1996; Yin et al., 1999.) The latter experiments used a monoclonal antibody called 3F5 raised against PTHrP. This antibody plays a central role in my research. It was developed by Dr. V. Grill in Australia and has been tested extensively (Rankin et al., 1995; 1997). Yin et al. (1999) demonstrated that this antibody could effectively block osteolytic metastases caused by human MDA-MB-231 breast cancer cells secreting PTHrP. Because of the slow development of bone metastases in prostate cancer, any treatment, such as administration of a therapeutic neutralizing antibody, would have to be repeated at least weekly over the course of many months. We decided instead to take a gene-therapy-related approach. We undertook to clone the light and heavy chain cDNAs encoding the 3F5 mAb, express both chains from a single DNA vector, and then stably express this DNA in either the animals or the

metastatic tumor cells themselves. In the first year of my training I have succeeded in cloning, expressing, and functionally assaying the 3F5 mAb. Stable clones of PC3 prostate cancer cells are now being prepared which secrete their own PTHrP-neutralizing antibody.

Recombinant secretion of cloned antibodies. Functional antibodies can be readily expressed in non-lymphoid cells (Biocca et al., 1990), which permits them to be delivered to specific anatomical sites *in vivo* (Piccioli et al., 1991). In the experiments presented here, the intent was to neutralize the extracellular effects of tumor-produced PTHrP on target cells in the bone microenvironment. This will be much more efficiently achieved by delivering the antibody to the tumor site in bone, rather than injecting animals with sufficiently large amounts of purified antibody to cause systemic neutralization of PTHrP. Alternative approaches to blocking tumor-produced PTHrP, such as anti-sense, could have multiple side effects by inhibiting intracellular versus extracellular functions of PTHrP (Dougherty et al., 1999). Intracrine actions of PTHrP, which may have growth effects on prostate cancer cells, could be mediated by nuclear targeting of intracellular PTHrP (Lam et al., 2000). Antibody secretion directed to the metastatic site should not interfere with confounding intracrine pathways involving PTHrP. Therefore we set out to clone the sequences encoding the 3F5 monoclonal antibody which neutralizes N-terminal PTHrP (Rankin et al., 1995). We have isolated the light and heavy chains of the mAb by PCR of RNA isolated from the hybridoma and primers against regions of conserved immunoglobulin sequence. After sequencing, the two chains were transferred into a bicistronic vector which expresses both chains on a single mRNA, separated by an internal ribosome reentry site (Duke et al., 1992). This permits equimolar biosynthesis of the two chains and avoids cellular toxicity associated with excess production of immunoglobulin heavy chains (Kolb & Siddell, 1997).

Methods:

RNA was isolated from the 3F5 hybridoma and reverse transcribed into oligo(dT)-primed cDNA using commercial kits (GIBCO-LifeTechnologies) according to the manufacturer's instructions. PCR kits were obtained from the same source. PCR products of the correct sizes were purified from agarose gels with a commercial kit (Qiagen), digested with the appropriate restriction enzymes and repurified by agarose gel electrophoresis. All DNA enzymes were obtained from New England BioLabs and used according to the manufacturer's recommendations. All PCR products were initially subcloned into pBluescript vector (Stratagene) and analyzed by restriction mapping, PCR, and automated DNA sequencing by the Center for Advanced DNA Technologies of this University. Sequence data were analyzed with MacVector 6.5 software (Oxford Molecular). Initial DNAs were ligated into the subcloning vector using polylinker sites in the plasmid corresponding to the restriction sites originally designed into the PCR primers. Ligated DNAs were transfomed into competent *E. coli* DH5α cells (GIBCO-LifeTechnologies) according to the manufacturer's instructions. The light chain was constructed as a 0.7kb *Pst* I to *Sal* I fragment and the heavy chain as a 1.4kb *Xba* I to *Bgl* II fragment. Plasmid inserts with the correct sequence were transferred into the bicistronic expression vector pVR1030, provided by Dr. P.M. Hobart (Vical, Inc, San Diego, CA), which carries a kanamycin resistance gene. The light chain was subcloned into pVR1030, and an initial subclone was isolated and mapped. The heavy chain was then subcloned into this intermediate to give the final plasmid, designated pVR3F5. DNAs were purified with Qiagen kits.

Expression DNA pVR3F5 was transfected into 293 cells (for transient assay) and into human breast MDA-MB-231 and human prostate PC3 cancer cell lines using Lipofectamine Plus kits (GIBCO-LifeTechnologies) according to the manufacturer's instructions. One day after transfection

the medium was changed to serum-free, and this medium was conditioned for 48hrs. Cell number was determined, and the media were assayed for mouse IgG content and for their ability to neutralize the bone-resorbing and osteoclastogenic activities of synthetic human PTHrP 1-34 (R&D Systems). Mouse marrow culture assays were carried out by standard means (Takahashi et al., 1988). Fetal rat long bone organ culture following standard procedures (Garrett et al., 1990) was kindly carried out by Mr. Paul Williams. Concentrations of mouse IgG in the serum-free media conditioned by human cells were determined by a capture ELISA, following standard procedures (Harlow & Lane, 1988). Standard mouse IgG, goat anti-mouse antibody conjugated to alkaline phosphatase, and alkaline phosphate substrate were from Sigma. Stable cell lines were selected by the addition of 500-800μg/ml G418 (GIBCO-LifeTechnologies) 48 hours after transfection with 9 parts pVR3F5 and 1 part pcDNA3 (to provide *neo* resistance).

Results:

Isolation of heavy chain clones. PCR primers were designed according to Coloma et al., 1992). A mixture of three 5' primers and one 3' primer was used. Mixed bases at a single position are indicated by parentheses, and the added *Xba* I and *Bgl* II sites are underlined:

g1 [5' GCTCTAGACACCATGG (AG)ATG(CG)AGCTG(TG)GT(CA)AT(CG)CTCTT 3']
g2 [5' GCTCTAGACACCATG(AG)ACTTCGGG(TC)TGAGCT(TG)GGTTTT 3']
g3 [5' GCTCTAGACACCATGGCTGTCTTGGGGCTGCTCTTCT 3']
g-3' [5' GAAGATCTTCATTTACCAGGAGAGTGGGAGAGGCTCTTCTCAGT 3']

PCR (30 cycles) under standard conditions gave a band of the expected size (1.4kb), which was subcloned into pBluescript and sequenced. The 1411 bp insert was derived from the g2 + g-3' primer pair and encoded a 464 amino acid heavy chain including signal peptide, shown in figure 1C. The DNA sequence includes internal sites for *Pst* I and *Sal* I, necessitating that the heavy chain be transferred into the expression vector only after the light chain had been subcloned via these two restriction sites.

Isolation of light chain clones. PCR primers were designed as above. A mixture of four 5' primers and one 3' primer was used. Mixed bases at a single position are indicated by parentheses, and the added *Pst* I and *Sal* I sites are underlined:

k1 [5' AACTGCAGACCATGGAGACAGACACACTCCTGCTAT 3']
k2 [5' AACTGCAGACCATGGATTTTCAAGTGCAGATTTTCAG 3']
k3 [5' AACTGCAGACCATGGAG(TA)CACA(GT)(TA)CTCAGGTCTTT(GA)TA 3']
k4 [5' AACTGCAGACCATG(GT)CCCC(AT)(GA)CTCAG(CT)T(CT)CT(TG)GT 3']
k-3' [5' GACGTCGACCTAACACTCATTCCTGTTGAAGCTCTTGACAATGGG 3']

PCR (30 cycles) under standard conditions gave a band of the expected size (0.75kb), which was subcloned into pBluescript and sequenced. Over a dozen clones were sequenced, all of which encoded a transcript previously reported to be expressed by the MOPC-21 tumor line, which is the partner in the cell fusion step of hybridoma generation (Coloma et al., 1992). This aberrant transcript has a frame shift in the kappa constant region and does not result in light chain protein synthesis. Attempts to reduce the amplification of this transcript by PCR optimization were unsuccessful. When tested pairwise, only the k1 + k-3' primer pair gave a PCR band of the correct size. We designed an aberrant chain-specific PCR primer whose 3' end annealed to the 4 extra bases found in this transcript (Coloma et al., 1992, figure 2). Individual clones of 3F5 cDNA amplified with k1 + k-3' and ligated as .75kb *Pst* I and *Sal* I fragments into pBluescript were isolated. Miniprep DNAs from white colonies on *lac* indicator plates were individually analyzed by PCR with k1 + k-3' for

presence of insert and with k1 + aberrant primer. Screening of over 50 colonies yielded two aberrant-negative candidates, which were designated K1 and K10. When sequenced, both encoded a nearly identical mouse IgG_1 kappa light chain without signal peptide. K1 and K10 differ by three N-terminal amino acid residues, designated 25-27, in Figure 1. The nucleotide sequences for the two cDNAs were identical elsewhere, suggesting that the amino acid differences represented a polymorphism. Near the 5' end of each clone was an *Nco* I restriction site which includes a Met codon (residue 23 in figure 1). The signal peptide of the aberrant V_κ transcript was isolated by PCR as a 75bp *Xba* I to *Nco* I fragment and ligated into the 5' ends of the K1 and K10 pBluescript subclones digested with the same two enzymes. The final pre-light chain protein sequences are shown in figures 1A and B.

Construction of pVR3F5. The light and heavy chains were cloned successively into the *Pst* I and *Sal* I sites and *Xba* I and *Bgl* II sites, respectively, of pVR1030. The map of the bicistronic vector is shown in figure 2. Transcription is driven by a CMV promoter. A major feature of the vector is the inclusion, between the cloning sites for the light and heavy chains, of an internal ribosome reentry sequence of viral origin, CITE, which permits two proteins to be translated in roughly equimolar amounts from a single mRNA transcript (Duke et al., 1992). The use of the bicistronic vector assures that the cloned antibody will be efficiently expressed, since the light and heavy chains should be synthesized in equal amounts. The vector carries a prokaryotic kanamycin resistance cassette but lacks a selectable marker for use in mammalian cells.

Expression of pVR3F5. The expression DNAs, in both K1 and K10 versions, were purified and tested for their ability to cause secretion of functional mouse IgG antibody. DNAs, versus control pVR1030, were transiently transfected into the human embryonal kidney cell line HEK 293. Serum-free conditioned media were assayed (figure 3) and found to contain about 50ng/ml mouse IgG. The procedure was repeated on a larger scale in T150 tissue culture flasks, which gave about 100ng/ml mouse IgG (figure 4). Both antibodies were effective, suggesting that the polymorphic differences between K1 and K10 did not affect binding to PTHrP. The K1 and K10 IgGs were concentrated by chromatography on Protein A/G minicolumns (Pierce ImmunoPure (A/G) IgG purification kit) according to the manufacturer's instructions. The concentrations of the purified proteins were determined with the IgG ELISA, and these materials were used in the subsequent assays.

Antibody produced from pVR3F5 blocks osteoclast formation in vitro. Osteoclast formation was assayed in cultures of mouse bone marrow (Takahashi et al., 1988) stimulated with either 10^{-8}M 1,25di(OH)vitamin D_3 or 20ng/ml PTHrP 1-34, which result in the formation of multinucleated osteoclast-like cells that stain positively for tartrate-resistant acid phosphatase [TRAP, assayed with a Sigma kit]. Figures 5-8 show that the pVR3F5-encoded IgG inhibited the formation of osteoclast-like cells in response to either stimulus. Both K1 and K10 antibodies appeared to be equivalently effective. Inhibition of the response to PTHrP was as expected. We do not have an explanation for the unanticipated inhibition of osteoclast-like cell formation in response to calcitriol.

Antibody produced from pVR3F5 blocks PTHrP-stimulated bone resorption in vitro. The addition of either K1 or K10 versions of the pVR3F5-derived antibody completely inhibited Ca^{45} release from labeled fetal rat long bones in culture (Figure 9).

Stable cell lines express 3F5 antibody. pVR3F5-k1 and pVR3F5-k10 were stably cloned into the human cancer lines PC3 (prostate) and MDA-MB-231 (breast), both of which secrete PTHrP and cause osteolytic metastases in a mouse model (Guise, 1997). Co-selection for G418 resistance resulted in the initial identification of about 20 positive clones out of 100 single-cell clones for each

cell line. These secreted up to 40ng/ml mouse IgG per 10^6 cells per 48hrs when first screened. Upon continuous passaging in tissue culture for 8 weeks every clone showed decreasing IgG secretion, despite continuing resistance to 500μg/ml G418. At the end of two months, no positive expressing clones remained. A parallel experiment selecting for secretion of a protein from a pcDNA3 plasmid construct [which carries G418 resistance and CMV-driven protein expression on the same DNA] showed no such loss of protein expression in either cell line. We concluded that the pVR1030 vector does not confer stable expression.

Recloning of bicistronic antibody cassette into stable vector. The light chain + 502bp CITE sequence [1.2kb *Pst* I to *Xba* I fragment] and the heavy chain sequence [1.4kb *Xba* I to *Bgl* II fragment] are presently being subcloned into pBluescript. In the case of the heavy chain subclone, the *Bgl* II sticky end will be ligated (irreversibly) to the *BamH* I site of the vector polylinker. The two fragments will be released from the pBluescript subclones as *EcoR* I to *Xba* I 1.2kb [pre-light chain + CITE] and *Xba* I to *Apa* I 1.4kb [heavy chain] fragments, which will then be sequentially ligated into the stable expression vector pcDNA3 (Clontech) digested with these same restriction enzymes. The product, pcDNA3F5, will be tested for transient IgG expression, then used to re-generate the stable clones in PC3 and MDA-MB-231 cells, as described above. pcDNA3 carries a *neo* resistance cassette which permits stable selection of mammalian cells with G-418. We plan to use the K10 version for subsequent experiments, for the sake of simplicity, since both K1 and K10 appear to encode equivalent neutralizing activities.

Summary of year 1:

In the first year of the proposal, I have been fully successful. I carried out tasks 1-6 of the total of 10 tasks proposed in the statement of work (p. 5 of the original application). Light and heavy chains of the 3F5 monoclonal antibody were cloned and sequenced and shown to direct efficient synthesis of antibody able to neutralize the actions of PTHrP to stimulate osteoclast formation and bone resorption-which are essential components of osteolytic metastases *in vivo*. My results were presented at the national meeting of the American Society of Bone and Mineral Research in Toronto.

Summary of year 2:

Task 5 of the statement of work was repeated, as described two paragraphs above, to place the sequences necessary for bicistronic expression of the 3F5 IgG light and heavy chains into a vector, pcDNA3, known to give stable clones when introduced into prostate cancer cells. Tasks 6 and 7, generation of stable clones in PC3 cells and determination of growth rates were carried out. I expected to be ready to carry out the central experiment of the proposal by January 2001, task 8, the *in vivo* metastasis experiment by injecting the stable PC3 cells into the left cardiac ventricle of male nude mice. However, over 90 individual stable clones, in several vectors were tested in vitro for continuing expression of the 3F5mAb. These clones used the different vectors described above. In addition a glutamine synthetase gene amplification cassette plus selection with the anti-metabolite methionine sulfoximine was tested (Cockett et al, 1990). All of these 90 clones were selected, from over 1000 primary clones in a series of experiments, for initial secretion of mouse IgG. Upon continued passaging, *none* of the clones maintained stable secretion of the mAb. Thus, we concluded that the antibody is negatively selected against in prostate cancer cells. This is compatible with data (published after our original proposal was submitted) suggesting that PTHrP is a necessary autocrine growth factor for prostate cancer cells such as PC3s (Tovar Sepulveda & Falzon, 2002; Dougherty et al, 1999). I therefore abandoned my original aim to carry out animal experiments with

PTHrP neutralization.

Alternate experimental approach to studying the role of PTHrP in prostate cancer bone metastases. . I used precisely the same approach used previously in our group with breast cancer cells (Yin et al, 1999) to reduce PTHrP production in PC3 cells. Stable clones were made expressing a dominant negative TGFbeta Type II receptor subunit and which had significantly reduced PTHrP secretion when challenged with 5ng/ml TGFbeta1. When tested in animals, these animals had *worse* rather than better bone metastases. My current experiments indicate that these effects are due to regulation of IGFBP-3 by TGFbeta. I include an abstract of my results.

Training Component:

I have found the training environment in the Division of Endocrinology to be highly useful and stimulating. I attend weekly Divisional data presentation meetings, at which I present my results to the faculty and post-doctoral fellows once a month. I attend a weekly Chirgwin-Guise joint laboratory meeting at which I present my results every week. I also participate in a biweekly bone cell biology journal club and attend a weekly endocrinology seminar program. Dr. Ian Thompson, Chief of Urology, has organized a prostate cancer research program with monthly meetings, which I attend. I participated in the one-day-long San Antonio Cancer Symposium in July (my Abstract this year was selected as the best of the 2002 meeting) and a scientific retreat to Corpus Christi in August of the Metastasis Program of the San Antonio Cancer Institute. In September 2001 I also participated in a 2-day training program in public presentation skills for scientists conducted by Brent Consulting Group and sponsored by my Division and Novartis Pharmaceuticals. This was of particular help to me since English is not my native tongue. Dr Chirgwin's laboratory provides full access in the laboratory to library facilities, e journals and literature databases, such as PubMed, through two computers. The substantial group of postdoctoral fellows interested in prostate cancer in general and bone metastasis in particular in the laboratories of Drs. Guise, Chirgwin, and Mundy provides a great deal of intellectual stimulation relevant to my scientific interests in the metastasis of prostate cancer to bone.

My more recent work in TGFbeta effects on cancer has enabled me to find a new position, in the laboratory of Dr. LhuZhe Sun in the Department of Cellular and Structural Biology. Dr Sun is an expert on the role of TGFbeta in cancer (Bandyopadhyay et al, 2002) and in prostate cancer in particular (Ahmad et al, 2002). I will begin working in Dr Sun's laboratory 10/01/2002.

KEY ACCOMPLISHMENTS

- Heavy chain of 3F5 monoclonal antibody cloned by PCR from hybridoma mRNA and sequenced.
- Kappa light chain (2 N-terminal polymorphic variants) cloned and sequenced, after elimination of large numbers of clones derived from aberrant light chain transcripts
- Secretory leader sequences added to two light chain cDNAs
- Light and heavy chains sequentially subcloned into pVR1030 bicistronic expression vector
- pVR1030/3F5 transiently expressed in 293 cells, resulting in secretion of mouse IgG1 protein.
- Secreted recombinant IgG neutralized PTHrP-stimulated Ca++ release from fetal rat long bones in organ culture
- Recombinant IgG blocked PTHrP-stimulated osteoclast formation stimulated by PTHrP in mouse bone marrow culture in vitro.
- Co-transfection of PC3 cells with pVR1030/3F5 and neo-resistance DNA resulted in initial clones secreting neutralizing antibody, when selected with the neo derivative G418. However, high level expression was not stably maintained, since the pVR1030/3F5 and neo-resistance DNAs were not physically linked to one another.
- Human glutamine synthetase cassette constructed and used for step-wise selection with gene amplification of resistant to methionine sulfoximine
- PC3 cells stably transfected with TGFbeta Type II receptor dominant negative construct and TGFbeta non-responsive clones with decreased PTHrP secretion selected.
- Clones tested in animal model of bone metastases. Analysis of animal data completed.
- PC3 clones screened for factor production by PCR and ELISA.
- IGFBP3 identified as factor important in increasing severity of bone metastases.

REPORTABLE OUTCOMES

- Sequence of 3F5 PTHrP-neutralizing monoclonal antibody determined. Will be desposited in public database upon publication or results.
- Modified prostate cancer animal model of bone metastasis established. To be reported at national meeting of American Society of Bone & Mineral Research in September 2002.
- New postdoctoral position in cancer research obtained

No patents or inventions

Animals were used in the final year (through protocol and funding of my mentor). 51 mice were used in bone metastasis experiments (50 were proposed in the original grant).

REFERENCES:

Ahmed MM, Alcock RA, Chendil D, Dey S, Das A, Venkatasubbarao K, Mohiuddin M, Sun L, Strodel WE, Freeman JW (2002). Restoration of transforming growth factor-beta signaling enhances radiosensitivity by altering the Bcl-2/Bax ratio in the p53 mutant pancreatic cancer cell line MIA PaCa-2. *J Biol Chem* **277**:2234-2246.

Asadi F, Farraj M, Sharifi R, Malakouti S, Antar S, Kukreja S (1996). Enhanced expression of parathyroid hormone-related protein in prostate cancer as compared with benign prostatic hyperplasia. *Hum Pathol* **27**: 1319-1323.

Bandyopadhyay A, Zhu Y, Malik SN, Kreisberg J, Brattain MG, Sprague EA, Luo J, Lopez-Casillas F, Sun LZ (2002). Extracellular domain of TGFbeta type III receptor inhibits angiogenesis and tumor growth in human cancer cells. *Oncogene* **21**:3541-3451.

Biocca S, Neuberger MS, Cattaneo A (1990). Expression and targeting of intracellular antibodies in mammalian cells. *EMBO J* **9**:101-108.

Carroll WL, Mendel E, Levy S (1988). Hybridoma fusion cell lines contain an aberrant kappa transcript. *Mol Immunol* **25**:991-995.

Coleman RE (1997). Skeletal complications of malignancy. *Cancer* **80**: 1588-1594.

Coloma MJ, Hastings A, Wims LA, Morrison SL (1992). Novel vectors for the expression of antibody molecules using variable regions generated by polymerase chain reaction. *J Immunol Methods* **152**:89-104.

Dougherty KM, Blomme EA, Koh AJ, Henderson JE, Pienta KJ, Rosol TJ, McCauley LK (1999). Parathyroid hormone-related protein as a growth regulator of prostate carcinoma. *Cancer Res* **59**: 6015-6022.

Duke GM, Hoffman MA, Palmenberg AC (1992). Sequence and structural elements that contribute to efficient encephalomyocarditis virus RNA translation. *J Virol* **66**:1602-1609.

Garrett IR, Boyce BF, Oreffo RO, Bonewald L, Poser J, Mundy GR (1990). Oxygen-derived free radicals stimulate osteoclastic bone resorption in rodent bone in vitro and in vivo. *J Clin Invest* **85**:632-639.

Goltzmann D (1997). Mechanisms of the development of osteoblastic metastases. *Cancer* **80**: 1581-1587.

Guise TA, Mundy GR (1998). Cancer and bone. *Endo Rev* **19**: 18-54.

Guise TA, Yin JJ, Taylor SD, Kumagai Y, Dallas M, Boyce BF, Yoneda T, Mundy GR (1996). Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis. *J Clin Invest* **98**: 1544-1549.

Guise TA (1997). Parathyroid hormone-related protein and bone metastases. *Cancer* **80**: 1572-1580.

Harlow E, Lane D (1988). Antibodies, A Laboratory Manual. Cold Spring Harbor Press, Cold Spring NY.

Iwamura M, di Sant'Agnese PA, Wu G, Benning CM, Cockett ATK, Deftos LJ, Abrahamsson P-A (1993). Immunohistochemical localization of parathyroid hormone-related protein in human prostate cancer. *Cancer Res* **53**: 1724-1726.

Iwamura M, Wu G, Abrahamsson P-A, Cockett ATK, Foss KA, Deftos LJ (1994). Parathyroid hormone-related protein: a potential autocrine growth regulator in human prostate cancer cell lines. *Urology* **43**: 675-679.

Jacobs SC (1983). Spread of prostatic cancer to bone. *Urology* **21**: 337-344.

Karp JE, Chiarodo A, Brawley O, Kelloff GJ (1996). Prostate cancer prevention: investigational approaches and opportunities. *Cancer Res* **56**: 5547-5556.

Kolb AF, Siddell SG (1997). Expression of a recombinant monoclonal antibody from a bicistronic mRNA. *Hybridoma* **16**:421-426.

Lam MH, Thomas RJ, Martin TJ, Gillespie MT, Jans DA (2000). Nuclear and nucleolar localization of parathyroid hormone-related protein. *Immunol Cell Biol* **78**:395-402.

Mincheff M, Tchakarov S, Zoubak S, Loukinov D, Botev C, Altankova I, Georgiev G, Petrov S, Meryman HT (2000). Naked DNA and adenoviral immunizations for immunotherapy of prostate cancer: A phase I/II clinical trial. *Eur Urol* **38**:208-217

Peehl DM, Edgar MG, Cramer SD, Deftos LJ (1997). Parathyroid hormone-related protein is not an autocrine growth factor for normal prostatic epithelial cells. *Prostate* **31**: 47-52.

Piccioli P, Ruberti F, Biocca S, Di Luzio A, Werge TM, Bradbury A, Cattaneo A (1991). Neuroantibodies: molecular cloning of a monoclonal antibody against substance P for expression in the central nervous system. *Proc Natl Acad Sci USA* **88**:5611-5615.

Rankin W, Murphy B, Grill V, Ho PWM, Moseley JM, Martin TJ (1995). Monoclonal antibody against parathyroid hormone-related protein with in vitro neutralizing activity. *Bone* **16**:205S.

Rankin W, Grill V, Martin TJ (1997). Parathyroid hormone-related protein and hypercalcemia. *Cancer* **80**: 1564-1571.

Restifo NP, Ying H, Hwang L, Leitner WW (2000). The promise of nucleic acid vaccines. *Gene Ther* **7**:89-92.

Takahashi N, Yamana H, Yoshiki S, Roodman GD, Mundy GR, Jones SJ, Boyde A, Suda T (1988). Osteoclast-like cell formation and its regulation by osteotropic hormones in mouse bone marrow cultures. *Endocrinol* **122**:1373-1382.

Tovar Sepulveda VA, Falzon M (2002). Parathyroid hormone-related protein enhances PC-3 prostate cancer cell growth via both autocrine/paracrine and intracrine pathways. *Regul Pept* **105**:109-120.

Yin JJ, Selander K, Chirgwin JM, Dallas M, Grubbs BG, Wieser R, Massague J, Mundy GR, Guise TA (1999). TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. *J Clin Invest* **103**:197-206.

APPENDIX:

1) Abstract describing initial work to be presented at 22nd annual meeting of the American Society of Bone and Mineral Research September 25th, 2000, Toronto, Canada: Abstract # 1209:

Paracrine Neutralization of Tumor-Produced PTHrP by Bicistronic Expression of Cloned Antibody Chains. XH Sun, X Li, WA Rankin, BG Grubbs, V Grill, TJ Martin, GR Mundy, M Gillespie, PM Hobart, TA Guise, JM Chirgwin. University of Texas Health Science Center at San Antonio, Peking Union Medical College Hospital, Beijing PRC, St Vincent's Institute of Medical Research, Melbourne Aus, and Vical, Inc, San Diego

2) Copy of abstract on most recent work on TGFbeta:

Blockade of TGFβ Signaling in Prostate Cancer Cells Exacerbates Osteolytic Metastases

X. Sun[1,2], S.-M. Käkönen[2], B.L. Hill[2], B.G. Grubbs[2], S. Burns[1], T.A. Guise[2], J.M. Chirgwin[1,2], [1]VA Research Service, and [2]Molec Med, U TX Hlth Sci Ctr, San Antonio, TX, USA

PC3 prostate cancer cells express abundant PTHrP and cause osteolytic metastases in nude mice. PTHrP production by PC3 cells is increased from 75 to 250pM/10^5cells/48h by 20ng/ml TGFβ1. We expected that making PC3 cells refractory to bone-derived TGFβ would decrease osteolytic metastases, as was observed with MDA-MB-231 breast cancer cells (Yin et al, J Clin Invest 103:197, 1999). Three stable clones of PC3 expressing a dominant negative TGFβ receptor construct pcDNA3 TβRIIΔcyt were isolated. 1B2 & 2B2 showed decreased PTHrP production basally and <75pM in response to TGFβ, while 2B4 had unchanged basal production and was stimulated only 1.8X. Growth rates were unchanged in vitro. When dominant negative clones were inoculated into the left cardiac ventricle of male nude mice (n=10), the animals died significantly sooner from osteolytic metastases than did the controls. Mice receiving 2B2 cells survived ~75d ($p<0.0001$ vs EV) and 1B2 & 2B4 groups survived ~150 days ($p<0.05$ vs empty vector, EV), at which time >75% of EV-inoculated animals were still alive. Media conditioned by the cell lines +/-5ng/ml TGFβ1 were analyzed for osteolytic factors. Dominant negative clones secreted less IL-11, VEGF, and PTHrP than control. These factors could not account for the results seen in vivo. Other factors with actions on bone cells or tumor growth were examined by RT-PCR +/-TGFβ. Factor mRNAs unchanged by TGFβ treatment and in clones vs control cells included: osteoprotegerin, RANK ligand, hepatocyte growth factor, tissue inhibitor of metalloproteinase-1, urinary plasminogen activator, plasminogen activator inhibitor-1, IL-18, and its binding protein. As reported (Hwa et al, Endocrine 6:235. 1997), insulin-like growth factor binding protein (IGFBP) 3 was stimulated by TGFβ. Its expression was decreased in the TβRIIΔcyt clones. IGFs are abundant components of bone matrix. When released by osteoclastic resorption, they could stimulate tumor cells in bone. This stimulation would normally be suppressed by IGFBP3 secreted in response to TGFβ, also released during resorption. The effects of TGFβ signaling blockade on bone metastases may differ between breast and prostate cancer models for 2 reasons: 1) PC3 prostate cells basally secrete very high amounts of PTHrP; even when TGFβ signaling is blocked; these are higher than those secreted by MDA-MB-231 cells and may be sufficient to sustain osteolysis. 2) TGFβ signaling may control prostate cancer cell growth in bone through a paracrine mechanism involving bone-derived IGFs and tumor-produced IGFBPs.